SUPPLEMENTAL MATERIALS

Endothelial-specific Transgenesis of TNFR2 Promotes Adaptive Arteriogenesis and Angiogenesis

Luo, Y.[1,2], Xu, Z.[2], Wan, T. [1,2], He, Y.[2], Jones, D.[2], Zhang, H.[2], and Min, W.[1,2,3]

[1]State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China. [2]Interdepartmental Program in Vascular Biology and Therapeutics, Yale University School of Medicine, 10 Amistad St, New Haven, CT 06520.

[3]Corresponding author: Dr. Wang Min, Interdepartmental Program in Vascular Biology and Therapeutics, Department of Pathology, Yale University School of Medicine, 10 Amistad St., 401B, New Haven, CT 06520. Tel: 203-785-6047; Fax: 203-737-2293; Email: wang.min@yale.edu.

MATERIALS AND METHODS

Animal protocol. Mice were housed in specific pathogen-free animal facilities according to guidelines from *the Office of Laboratory Animal Welfare at the National Institute of Health*, and all experimental procedures were approved by *the institutional animal care and use committees* at Yale University.

Creation of EC-specific transgenic mice expressing TNFR2 (TNFR2-TG). The human TNFR2 cDNA with a Myc-tag sequence at 3'-end was inserted into the *EcoR*I and *Xba*I sites of pVE-pA vector [1] between the VE-cadherin promoter and bGH pA to obtain pVE-TNFR2 plasmid. The plasmid was linearized with *Xho*I digestion and the pronuclear injection was performed at Yale Transgenic Core. The founder were identified by PCR of tail DNA with a 5' primer of TNFR2 and 3' primer of Myc. TNFR2-TG mice were backcrossed with C57BL/6 mice for more than 6 generations before experiments. All experiments were performed with heterozygous TNFR2-TG mice and their non-TG littermates as controls.

TNFR2-KO (strain name B6.129-*tnfrsflb*) were initially purchased from Jackson Laboratory (Bar Harbor, Maine) and subsequently bred with C57BL/6 for at least six generations prior to experiments. Mice were confirmed by genotyping with specific primers suggested by the Vendor. TNFR2-KO/TG mice were obtained by mating of TNFR2-TG with TNFR2-KO and the KO/TG mice express TNFR2 only in vascular EC.

Immunohistochemistry for transgene expression. 8 to 10-week-old TNFR2-TG or WT littermates were perfused with PBS for 5 min and then with 4% paraformaldehyde for 5 min at physiological temperature and pressure. Blood vessels were then harvested and diffusion-fixed overnight at 4°C followed by dehydration in 30% sucrose overnight at 4°C. The vessels were then embedded, and 5-μm sections were stained for anti-Myc antibody (Roche) or anti-TNFR2 (rabbit polyclonal, Abcam). Endothelium was visualized by goat anti-CD31 antibody (Santa Cruz).

In vivo Angiogenesis Models. All animal studies were approved by the institutional animal care and use committees of Yale University. Wild type C57BL/6 mice were mated with TNFR2-TG to obtain the transgene-negative (WT) and transgene-positive (TG) littermates. Similarly, TNFR2-KO and TNFR2-KO/TG were mated to obtain littermates of KO and KO/TG. Age and sex-matched 8-12 week old male of WT, TNFR2-TG, TNFR2-KO and TNFR2-KO/TG mice were used for all experiments.

Mouse hindlimb ischemia model was performed as described recently [2-4]. Briefly, following anesthesia (79.5 mg/kg ketamine, 9.1 mg/kg; xylazine), the left femoral artery was exposed under a dissection microscope. The proximal of femoral artery and the distal portion of saphenous artery were ligated. All branches between these two sites were ligated or cauterized, and arteriectomy was performed. Sham operation is without femoral artery ligation but skin incision. Blood flow was measured by PeriFlux system with Laser Doppler Perfusion Module (LDPU) unit (Perimed, Inc. North Royalton, OH). Deep measurement probe was placed directly on gastrocnemius muscle to ensure a deep muscle flow measurement. Ischemic and non-ischemic limb perfusion was measured pre-, post-surgery, 3 days, 2 weeks and 4 weeks after surgery. The final blood flow values were expressed as the ratio of ischemic to non-ischemic hind limb perfusion.

Post-contraction hyperemia pre- and post- ischemia. After anesthesia, mice were placed on a heated pad. The adductor muscle group and gastrocnemius muscle were exposed by a middle line incision of the limb. After baseline gastrocnemius blood flow was measured, adductor muscles were stimulated with 2 electrodes at 2Hz, 5mA by using electrostimulator for 2 min. Blood flow was taken and recorded by MacLab Chart software (ADIstruments, Grand Junction, CO) during stimulation and for 10 minutes post-stimulation.

Microfil Perfusion. 4 weeks after femoral ligation, mice were anesthetized and perfused with 20 ml of 37°C PBS plus 10 units/ml heparin at a flow rate of 10–15 ml/min through the left ventricle. To analyze

the vasculature after onset of ischemia, we employed a silicone-based contrast formulation (Microfil, FlowTech Inc., USA) to optimize visualization of the vascular structures in mice. Arterial growth was quantitated as ratio of diameters (left/right).

Histology and Immunohistochemistry. Mice were sacrificed at indicated times and tissues were perfused in situ with PBS and then fixed with 10% buffered formalin. The sections were paraffin embedded, sectioned in 5 μM, and stained with hematoxylin & eosin. Tissue sections were also stained with certain antibodies (e.g., anti-CD31, anti-α-SMA, anti-TNFR2 and anti-CD45) as described previously [2, 3]. Bound primary antibodies were detected using avidin-biotin-peroxidase (NovaRed™ peroxidase substrate kit, Vector Laboratories, Burlingame, CA). Pictures from 4 random areas of each section, and 5 sections per mice were taken using a Kodak digital camera mounted on a light microscope (40x objective). Images were quantified using the Matlab software (The Math Works, Inc. Natick, MA) as described recently [2, 3]. TUNEL assay was performed according to the instruction provided by the Manufacturer's protocol (Roche) [3].

Gene expression in ischemic muscle. Total RNA of lower limb muscles was isolated by using phenol/chloroform and isolated using RNeasy kit with DNase I digestion (Qiagen, Valecia, CA). Reverse transcription was done by standard procedure (Super Script First-Strand Synthesis System, Qiagen) using 1μg total RNA. Quantitative real-time PCR was performed by using iQ SYBR Green Supermix on iCycler Real-Time Detection System (Bio-Rad Laboratories, Inc. Hercules, CA). Specific primers for mouse TNFR2, VEGFR2, Bmx, TNF, TNFR1, TRAF2 and 18S ribosomal RNA were described previously [2-4]. Relative amount of mRNA in mouse lower limb muscle 3 days and 2 week post-ischemia was quantified.

Aortic-Ring Assay. The thoracic aorta was dissected from mice (8–10 weeks old) and cut into cylindrical, 3-mm-long segments. The rings were suspended by two tungsten wires mounted in a vessel

myograph system (Danish Myotechnologies, Aarhus, Denmark). The aortas were bathed in oxygenated Krebs buffer and submitted to a resting tension of 9.8 mN. After 60 min of equilibration with frequent washings, concentration response curves for phenylephrine (PE) were generated to determine vasoconstrictor responses. To study vasodilator and L-nitro arginine methyl ester (L-NAME) responses, the rings were preconstricted with a submaximal concentration of PE, and acetylcholine (ACh) (10^{-9} to 10^{-5} M), sodium nitroprusside (SNP) (10^{-9} to $3x10^{-7}$ M), or L-NAME (100 μM) was injected at the plateau of the PE-induced contraction.

Bone marrow transplantation (BMT). Bone marrow transplantation (BMT) was performed as described previously [2]. Briefly, lethally irradiated mice (irradiation 2x 5.5 Gy within 3h) were transplanted with cells derived from donor mice, and bone marrow cells from TNFR2-KO or TNFR2-KO/TG mice were harvested by flushing femur. Red blood cells were removed by lysis using ammonium chloride and subsequent washing with PBS. Cells were counted and $1x10^5$ cells were injected into the tail vein of the TNFR2-KO recipient. Successful BMT was controlled by genotyping of TNFR2 gene six weeks after BMT. The mice were then subjected to femoral artery ligation as described in the methods.

Mouse EC culture. Mouse EC isolation from lung tissues was performed as we described previously [3, 4]. For immuno-selection, 10 μl beads (per T-75 of mouse lung cells) were washed with 1 ml of buffer A (PBS +2% FBS) for 3 times and resuspended in 100 μl of buffer A. 10 μl (10 μg) of anti-mouse ICAM-2 or 10 μl (10 μg) of PECAM-1 were added and rocked at 4°C for 2 hrs. Beads were washed for 3 times and resuspended in 160 μl of buffer A. Confluent mouse lung cells cultured in a T-75 flask were placed at 4°C for 5 min and incubated with the beads at 4°C for 1 hr. Cells were then washed with warm PBS and treated with 3 ml of warm Trypsin/EDTA. When cells were detached, 7 ml of growth media were added. An empty 15-ml tube in the magnetic holder was placed on the holder and the cell suspension

(~10 ml) was added slowly by placing the pipette on the wall of the tube so that the cells pass through the magnetic field. Cells were incubated for 5 min, and the media was carefully aspirated. The 15-ml tube was removed from the magnetic holder and the beads/cells were resuspended in 10 ml of media. The selected cells were plated on 0.2% gelatin-coated flasks and cultured for 3-7 days. When the cells were confluent, another round of immunoselection was repeated.

Statistical analysis. All data are expressed as mean ± standard error of the mean (SEM). Statistical differences were measured by either Student's T-test, one or two-way analysis of variance followed by Bonferonni post-hoc test. A value of $p < 0.05$ was considered as statistically significant.

SUPPLEMENTAL FIGURE AND FIGURE LEGENDS

Endothelial-specific Transgenesis of TNFR2 Promotes Adaptive Arteriogenesis and Angiogenesis

Luo, Y.[1,2], Xu, Z.[2], Wan, T. [1,2], He, Y.[2], Jones, D.[2], Zhang, H.[2], and Min, W.[1,2]

[1]State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China. [2]Interdepartmental Program in Vascular Biology and Therapeutics, Yale University School of Medicine, 10 Amistad St, New Haven, CT 06520.

Supplemental Fig.I

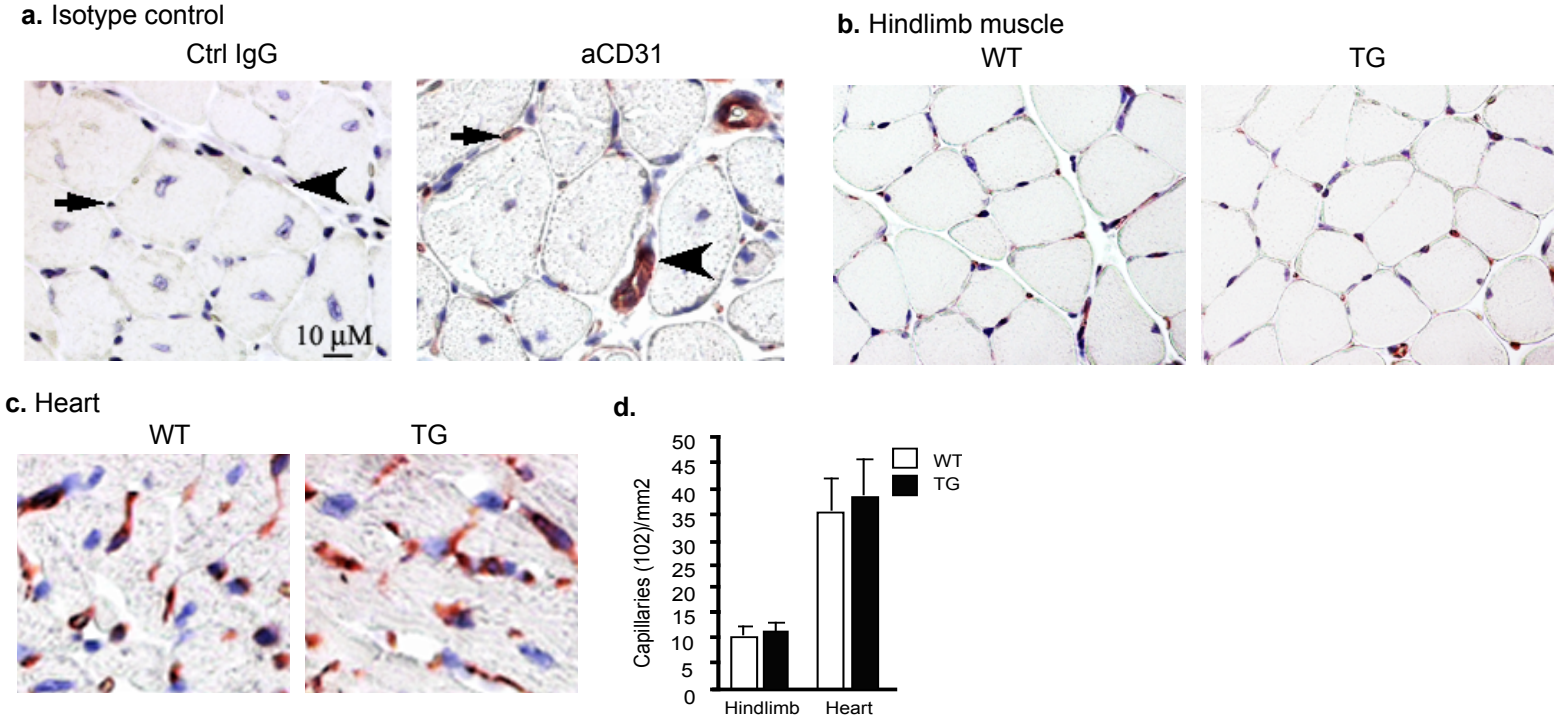
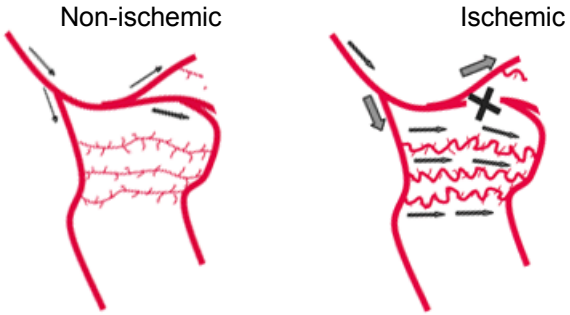


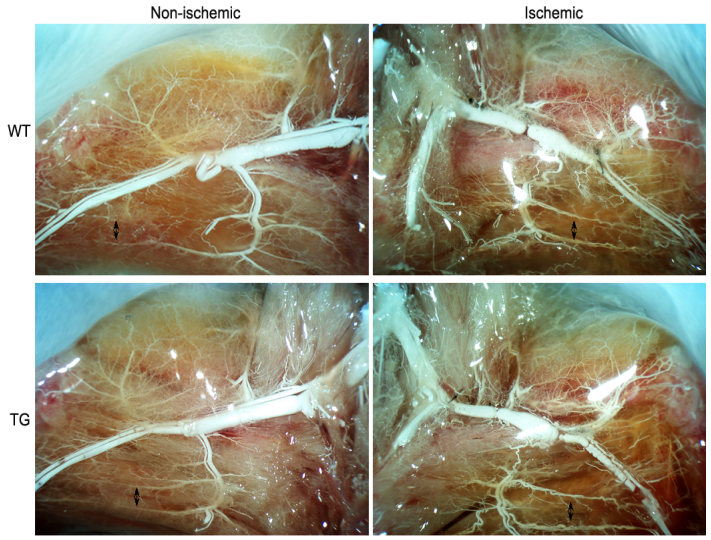
Fig.I. TNFR2 transgene expressed in vascular endothelium has no effect on the basal capillary density of tissues. **a**. The specificity of anti-CD31. Hindlimb tissue sections were immunstained with a goat IgG isotype control or anti-CD31. Arterioles are indicated by arrowheads and capillaries are indicated by arrows. **b-c**. Basal capillary density in hindlimb muscle and heart was immunostained with CD31. Representative images of CD31 staining in muscle and heart. **c**. Quantification of capillaries (number/mm^2 muscle area. Data from 3 sections of each mouse muscle tissue are shown in graphics and n=3 for each strain (total 9 sections). *, p<0.05.

Supplemental Fig.II

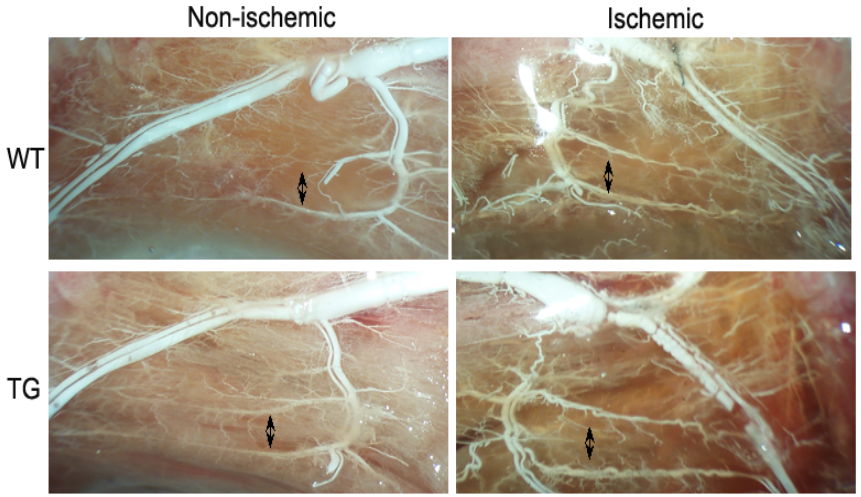
a: Casting- low power



b: Casting- low power



c: High power



d.

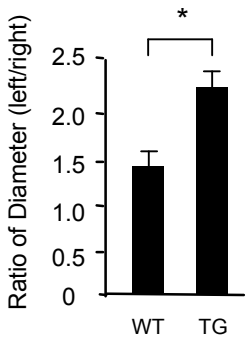
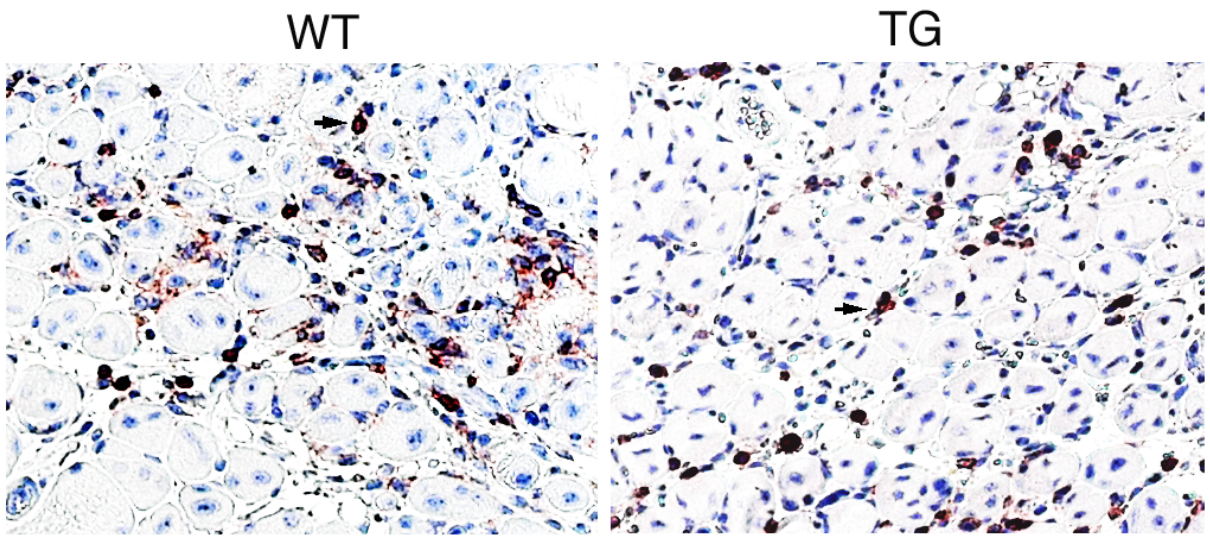


Fig.II. TNFR2 transgene increases ischemia-induced arteriogenesis. **a.** Diagram of blood flow changes after occlusion of the femoral artery in the hind limb. After occlusion of the femoral artery (X, left panel), blood flow (arrows) follows the gradient between high pressures proximal to the occlusion site and very low distal pressures. Preexisting collateral arterioles are recruited. **b-c**. Arteriogenesis was determined by Microfil casting analyses. 4 weeks after femoral ligation, mice were anesthetized and subjected to microfil perfusion. A low and high power are shown in a and b, respectively. Collateral artery growth is indicated by arrows. **d**. Arterial growth was quantified as a ratio of diameters (left/right) and n=5 for each strain. *, $p<0.05$.

Supplemental Fig.III

a. Infiltration (CD45 staining)



b.

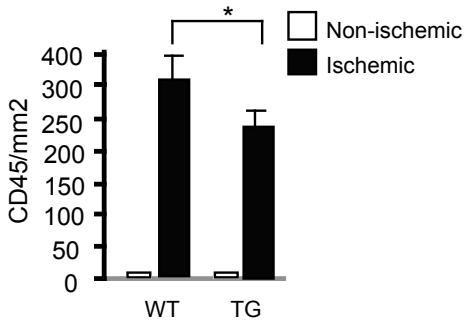
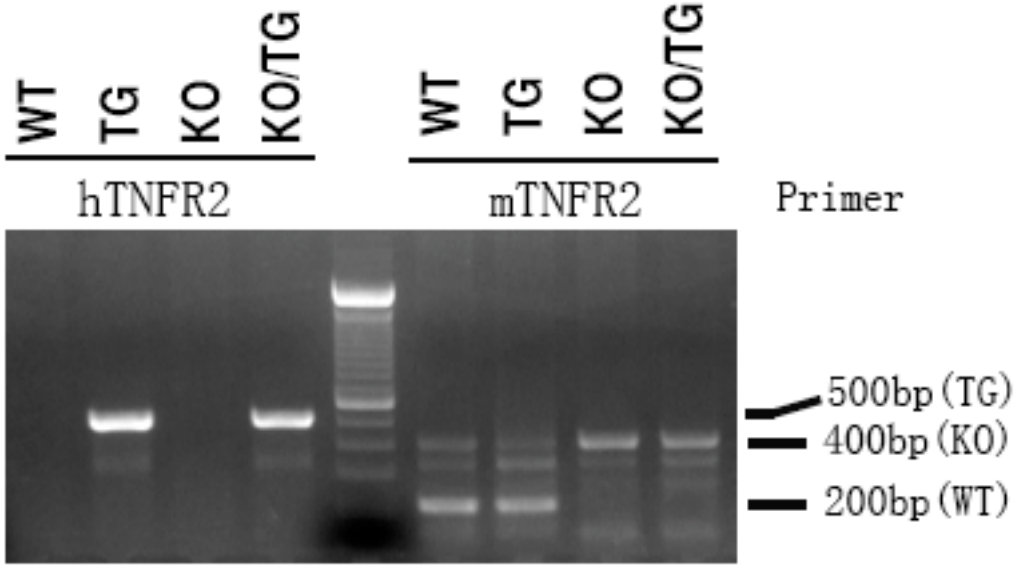


Fig.III. Effects of TNFR2 transgene on ischemia-induced infiltration. WT and TNFR2-TG mice were subjected to ischemia ligation, hindlimb tissues were harvested on day 7. Recruitment of leukocytes in response to ischemia was determined by anti-CD45. **a**. Representative images of CD45 staining in ischemic and non-ischemic hindlimbs of WT and TNFR2-TG mice are shown. **b**. CD45-positive cells were quantified as number/mm^2 muscle area. *, $p<0.05$.

Supplemental Fig.IV

a: Genotyping



b: TNFR2 staining

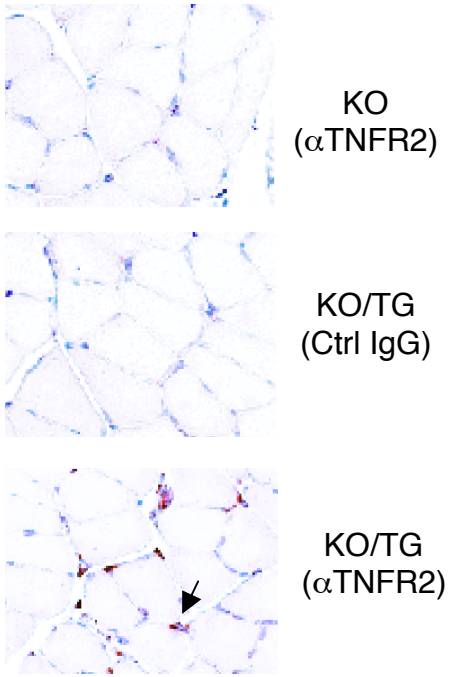


Fig.IV. Characterization of TNFR2-KO/TNFR2-TG mice. a. Genotyping of TNFR2-KO/TNFR2-TG mice. TNFR2-TG mice were mated with TNFR2-KO mice (both are C57BL/6 background) to obtain TNFR2-KO/TNFR2-TG (KO/TG) mice. Genomic DNA of mouse tails was genotyped by PCR with specific primers for the human TNFR2 transgenic fragment and the endogenous mouse TNFR2. **a**. A representative genotyping of WT, TG, KO and KO/TG is shown. **b.** Immunostaining of TNFR2. Muscle hindlimb tissues from TNFR2-KO and TNFR2-KO/TNFR2-TG mice were immunostained with anti-TNFR2. A rabbit IgG isotype was used as a control. Positive staining of TNFR2 in capillary is detected in KO/TG but not in KO mice.

Supplemental Fig.V

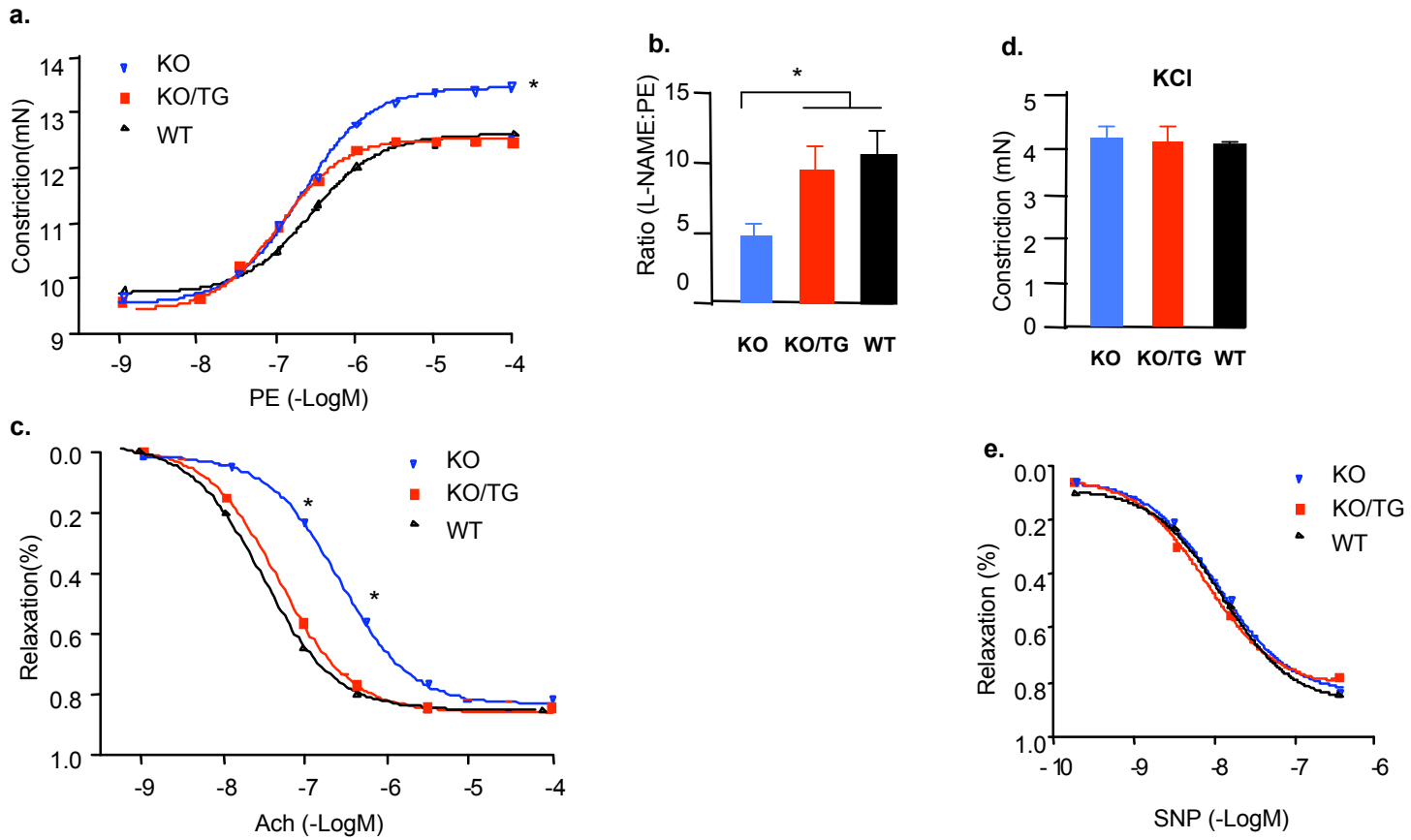
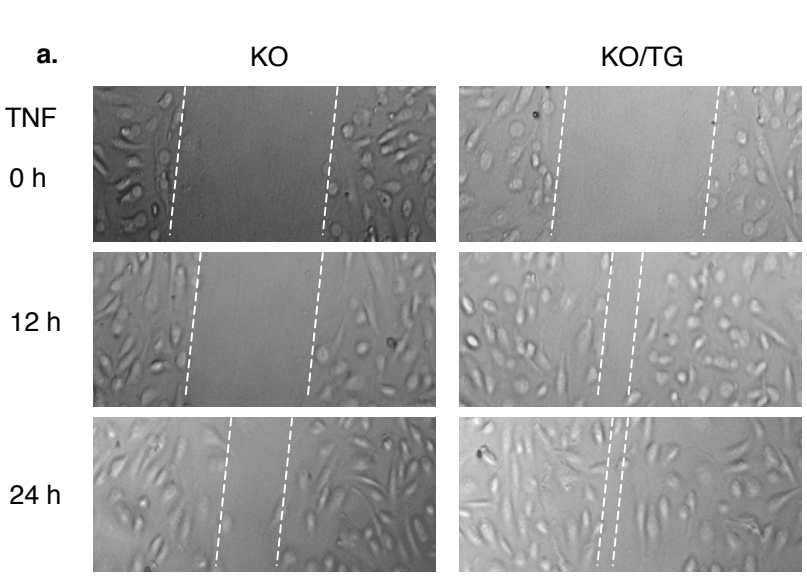
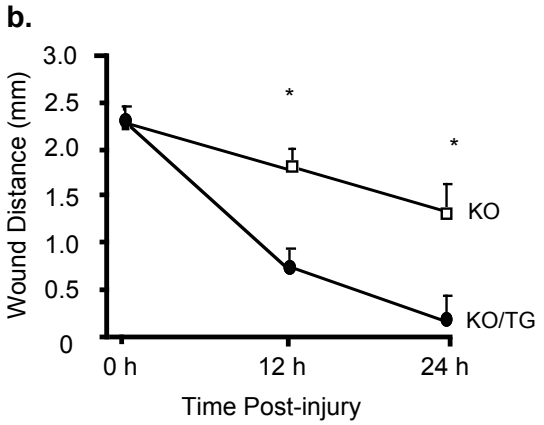


Fig. V. TNFR2-KO mice show reduction of basal NO release in aortic endothelium but TNFR2-TG restored the basal NO activity. Aortas from WT, KO and KO/TG (n=3 for each group) were used for aortic ring assays. **a**. PE responses. Aortas were contracted with PE at a full range of doses (10^{-9}-10^{-4} M). **b**. Basal NO activity. Aortic rings were incubated with a NOS inhibitor L-NAME to remove basal NO synthesis and then contracted with PE as in **a**. Ratio of EC_{50} in response to PE in the presence of L-NAME to PE in the absence of L-NAME. **c**. Ach responses. Aortic rings were precontracted with PE and then relaxed with Ach at a full range of doses (10^{-9}-10^{-4} M). % of relaxation is shown. **d**. Vessel constriction in response to KCl. Aortic rings were contracted with 50 mM of KCl. **e**. TNFR2 expression had no effects on vessel relaxation in response to the NO donor drug SNP. Aortic rings were incubated with L-NAME to remove basal NO synthesis followed by a precontraction with PE**,** and were then relaxed with SNP at a full range of doses (10^{-9}-10^{-6} M). All data are mean±SEM, with n=3 animals and eight aortic rings per animal, *, p<0.05.

Supplemental Fig.VI





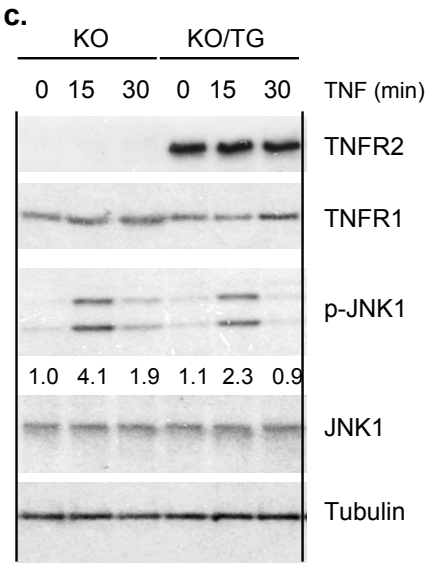


Fig.VI. **TNFR2 expression increases EC migration with no effects on TNFR1-dependent JNK activation**. **a-b**. Effects of TNFR2 transgene on EC migration. Mouse microvessel EC (MEC) from lung were isolated from KO and TNFR2-KO/TG mice, and TNF-induced EC migration was determined in a monolayer injury model by treating cells with TNF (1 ng/ml) for 0, 12 and 24 h. The wound areas were marked by white lines (**a**). Data are mean ±SEM of the two triplicates from two independent experiments. *, $p<0.05$ (**b**). **c**. Effects of TNFR2 transgene on TNFR1-dependent JNK activation. MEC were treated with TNF (10 ng/ml) for indicated time. JNK1 activation was determined by Western blot with a phospho-JNK1 specific antibody. Total JNK1 as well as expression of TNFR2, TNFR1 and β-tubulin were determined by Western blot with respective antibodies. The relative levels of phospho-JNK are quantified, with untreated TNFR2-KO EC as 1.0 (**e**). Similar results were obtained in two additional experiments.

References

1. Zhang H, Luo Y, Zhang W, He Y, Dai S, Zhang R, Huang Y, Bernatchez P, Giordano FJ, Shadel G, Sessa WC, Min W. Endothelial-specific expression of mitochondrial thioredoxin improves endothelial cell function and reduces atherosclerotic lesions. *Am J Pathol.* 2007;170:1108-1120.
2. He Y, Luo Y, Tang S, Rajantie I, Salven P, Heil M, Zhang R, Luo D, Li X, Chi H, Yu J, Carmeliet P, Schaper W, Sinusas AJ, Sessa WC, Alitalo K, Min W. Critical function of Bmx/Etk in ischemia-mediated arteriogenesis and angiogenesis. *J Clin Invest.* 2006;116:2344-2355.
3. Luo D, Luo Y, He Y, Zhang H, Zhang R, Li X, Dobrucki WL, Sinusas AJ, Sessa WC, Min W. Differential functions of tumor necrosis factor receptor 1 and 2 signaling in ischemia-mediated arteriogenesis and angiogenesis. *Am J Pathol.* 2006;169:1886-1898.
4. Zhang H, He Y, Dai S, Xu Z, Luo Y, Wan T, Luo D, Jones D, Tang S, Chen H, Sessa WC, Min W. AIP1 functions as an endogenous inhibitor of VEGFR2-mediated signaling and inflammatory angiogenesis in mice. *J Clin Invest.* 2008;118:3904-3916.